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Regulation of Cloned ATP-sensitive K Channels by Phosphorylation, MgADP, and Phosphatidylinositol Bisphosphate (PIP₂)

A Study of Channel Rundown and Reactivation

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abstract Kir6.2 channels linked to the green fluorescent protein (GFP) (Kir6.2-GFP) have been expressed alone or with the sulfonylurea receptor SUR1 in HEK293 cells to study the regulation of K_{ATP} channels by adenine nucleotides, phosphatidylinositol bisphosphate (PIP₂), and phosphorylation. Upon excision of inside-out patches into a Ca²⁺- and MgATP-free solution, the activity of Kir6.2-GFP+SUR1 channels spontaneously ran down, first quickly within a minute, and then more slowly over tens of minutes. In contrast, under the same conditions, the activity of Kir6.2-GFP alone exhibited only slow rundown. Thus, fast rundown is specific to Kir6.2-GFP+SUR1 and involves SUR1, while slow rundown is a property of both Kir6.2-GFP and Kir6.2-GFP+SUR1 channels and is due, at least in part, to Kir6.2 alone. Kir6.2-GFP+SUR1 fast phase of rundown was of variable amplitude and led to increased ATP sensitivity. Excising patches into a solution containing MgADP prevented this phenomenon, suggesting that fast rundown involves loss of MgADP-dependent stimulation conferred by SUR1. With both Kir6.2-GFP and Kir6.2-GFP+SUR1, the slow phase of rundown led to further increase in ATP sensitivity. Ca²⁺ accelerated this process, suggesting a role for PIP₂ hydrolysis mediated by a Ca²⁺-dependent phospholipase C. PIP₂ could reactivate channel activity after a brief exposure to Ca²⁺, but not after prolonged exposure. However, in both cases, PIP₂ reversed the increase in ATP sensitivity, indicating that PIP₂ lowers the ATP sensitivity by increasing P_o as well as by decreasing the channel affinity for ATP. With Kir6.2-GFP+SUR1, slow rundown also caused loss of MgADP stimulation and sulfonylurea inhibition, suggesting functional uncoupling of SUR1 from Kir6.2-GFP. Ca²⁺ facilitated the loss of sensitivity to MgADP, and thus uncoupling of the two subunits. The nonselective protein kinase inhibitor H-7 and the selective PKC inhibitor peptide 19-36 evoked, within 5–15 min, increased ATP sensitivity and loss of reactivation by PIP₂ and MgADP. Phosphorylation of Kir6.2 may thus be required for the channel to remain PIP₂ responsive, while phosphorylation of Kir6.2 and/or SUR1 is required for functional coupling. In summary, short-term regulation of Kir6.2+SUR1 channels involves MgADP, while long-term regulation requires PIP₂ and phosphorylation.

key words: Kir6.2 • phosphatidylinositol bisphosphate • MgADP

INTRODUCTION

ATP-sensitive K (K_{ATP}) channels are comprised of a pore-forming protein of the Kir6 family and the sulfonylurea receptor SUR. Patch-clamp recordings of both native and cloned K_{ATP} channels show decay of channel activity upon patch excision. This phenomenon, referred to as “rundown,” is avoided or even reversed by addition of MgATP, but not MgAMPPNP. It was initially postulated that channel dephosphorylation was responsible for rundown (Misler et al., 1986; Ohno-Shosaku et al., 1987; Ribalet et al., 1989). More recent studies, however, have shown that native and cloned K_{ATP} channels are reactivated by phosphatidylinositol bisphosphate (PIP₂)¹ after rundown (Furukawa et al., 1996;

Hilgemann and Ball, 1996; Fan and Makielski, 1997, 1999; Baukowitz et al., 1998; Shyng and Nichols, 1998). These findings, together with the observation that very high concentrations of wortmannin, an inhibitor of PIP₂ production, prevents K_{ATP} channel reactivation by MgATP (Xie et al., 1999) have led to the interpretation that reactivation by MgATP is due to PIP₂ re-synthesis. In addition, elevation of internal Ca²⁺ greatly accelerates rundown and MgATP reverses this effect (Ohno-Shosaku et al., 1987; Furukawa et al., 1996), consistent with the idea that Ca²⁺-induced rundown involves activation of Ca²⁺-dependent phospholipase C that hydrolyzes PIP₂ (Hilgemann and Ball, 1996).

However, it has also been noted that prolonged periods of rundown led to irreversible channel closure, so that limited or no reactivation occurred with reexposure to MgATP or PIP₂ (Furukawa et al., 1996). This indicates that rundown involves other factors besides PIP₂ hydrolysis. Protein kinase-mediated phosphorylation is one possible candidate, since protein kinase C inhibi-

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¹Abbreviations used in this paper: GFP, green fluorescent protein; PIP₂, phosphatidylinositol bisphosphate.

tion has been shown to increase ATP sensitivity (Hu et al., 1996; Light et al., 1996) similar to rundown (Deutsch et al., 1994; Terzic et al., 1994; Baukrowitz et al., 1998). In addition, we observed that rundown occurred more rapidly in the presence of the sulfonylurea receptor, SUR1, than in its absence (John et al., 1998), suggesting that SUR1 regulates the rundown process. Since SUR1 confers MgADP sensitivity to cloned K_{ATP} channels (Tucker et al., 1997; John et al., 1998) and rundown is accompanied by a loss of sensitivity to nucleotide diphosphates (Findlay, 1987; Bokvist et al., 1991; Terzic et al., 1994), binding of MgADP to SUR could play a role.

To assess the roles of PIP_2 , MgADP and phosphorylation in controlling the activity and rundown of K_{ATP} channels, we performed excised inside-out patch-clamp experiments of the Kir6.2-GFP+SUR1 complex and the pore-forming protein Kir6.2-GFP alone. Specifically, we investigated how these factors regulate the ATP sensitivity of the two channel types and the coupling between SUR1 and Kir6.2. A model is proposed to account for Kir6.2+SUR1 and Kir6.2 channel regulation by MgADP, PIP_2 , and protein kinases.

MATERIALS AND METHODS

The techniques used in the present studies for cDNA expression and patch clamp recording have been recently described in detail (John et al., 1998) and are only briefly outlined here.

Molecular Biology and cDNA Expression in HEK293 Cells

HEK293 cells were transfected with cDNA for Kir6.2 linked to green fluorescent protein (GFP) at the COOH terminus (Kir6.2-GFP). All wild-type cDNAs were subcloned into the vector pCDNA3amp (Invitrogen). cDNAs used to make the GFP chimeras were subcloned into the pEGFP vector. Both vectors used the cytomegalovirus promoter. The transfections were carried out using the calcium phosphate precipitation method (Graham and van der Eb, 1973). Expression of proteins linked to GFP was detected as early as 12 h after transfection. Patch-clamp experiments were started ~30 h after transfection. HEK293 cells were cultured in DMEM high glucose medium supplemented with 10% (vol/vol) fetal calf serum, penicillin (100 U/ml), streptomycin (100 U/ml), and 2 mM glutamine and divided once a week by treatment with trypsin.

Patch Clamp Methods

Currents were recorded in HEK293 cells using the inside-out patch-clamp configuration, with the pipette solution containing (mM): 140 KCl, 10 NaCl, 1.1 $MgCl_2$, 10 HEPES, pH 7.2 with KOH. The bath solution consisted of (mM): 140 KCl, 10 NaCl, 1.1 $MgCl_2$, 10 HEPES, 5 EGTA, 0.5 $CaCl_2$, pH 7.2 with KOH. ATP was added directly to the bath as MgATP. PIP_2 (Boehringer) was sonicated immediately before use. The protein kinase inhibitor H-7 and the phosphatase inhibitor okadaic acid were obtained from Calbiochem. The specific protein kinase C inhibitor peptide 19-36 was purchased from Peninsula Laboratories.

The data, filtered at 2 kHz with an eight-pole Bessel filter, was recorded with a patch clamp amplifier (EPC 7; List) and recorded on videotape at a fixed frequency of 44 kHz after digitiza-

tion with a digital audio processor. For analysis, the data was sampled at a rate of 5.5 kHz. When discrete current steps could be resolved, channel activity was expressed as NP_o . To express channel activity as a function of ATP concentration, NP_o was estimated at each ATP concentration from data samples of 15 s duration. Since channel activity varied widely from patch to patch, NP_o values were normalized to values measured in the absence of ATP. When single channels could not be resolved, steady state current values were used instead of NP_o to assess the effects of adenine nucleotides and other interventions on channel activity.

Single-Channel Data Analysis and Fit of ATP Dose-dependent Curves

Analysis of single-channel data was carried out using the approach described in detail previously (Ribalet et al., 1989), which consists of fitting the open- and closed-time histograms with a sum of exponentials. Since in most cases more than one level of channel opening was observed, we could not construct closed-time histograms. However, open-time histograms could be constructed, since membrane patches with low activity at the second level of opening could be obtained.

RESULTS

Spontaneous Rundown of Kir6.2-GFP+SUR1 Occurs in Two Phases with Distinct Properties

Kir6.2-GFP+SUR1 channel activity increased rapidly when inside-out patches were excised into a MgATP-free bath solution with Ca^{2+} concentration $<10^{-7}$ M. Their activity then typically ran down in a fast and a slow phase (Fig. 1 A). The amplitude of the fast phase was variable. For instance, in six patches, channel activity decreased by 50% within 105 ± 42 s and was inhibited by $>80\%$ from the initial level by the start of the slow phase. In contrast, in seven other patches, there was no significant phase of fast rundown. In the majority of patches ($n = 38$), however, the amplitude of the fast phase varied between these two extremes. After fast rundown, channel activity declined more slowly, leading to nearly complete inhibition after several hours.

Fast rundown. The amplitude of the fast phase of rundown correlated with the channel ATP sensitivity. In six patches with a pronounced fast phase of rundown, the concentration of ATP causing half-maximal channel inhibition (IC_{50}) after fast rundown averaged 23 μ M, whereas in four patches without a significant fast phase of rundown, the IC_{50} averaged 210 μ M. MgADP reactivated the ATP-inhibited channels regardless of whether the initial ATP sensitivity was low or high (Fig. 1 B), and sulfonylureas blocked channel activity (not shown), indicating functional coupling between Kir6.2 and SUR1. Thus, in two patches with pronounced fast rundown and with an averaged IC_{50} for ATP-dependent inhibition of 15 μ M, MgADP reactivated ATP-inhibited channels to a similar extent (67%) to that in the absence of prominent fast rundown.

We observed that MgADP was a powerful inhibitor of the fast phase of rundown. In 12 of 14 patches tested,

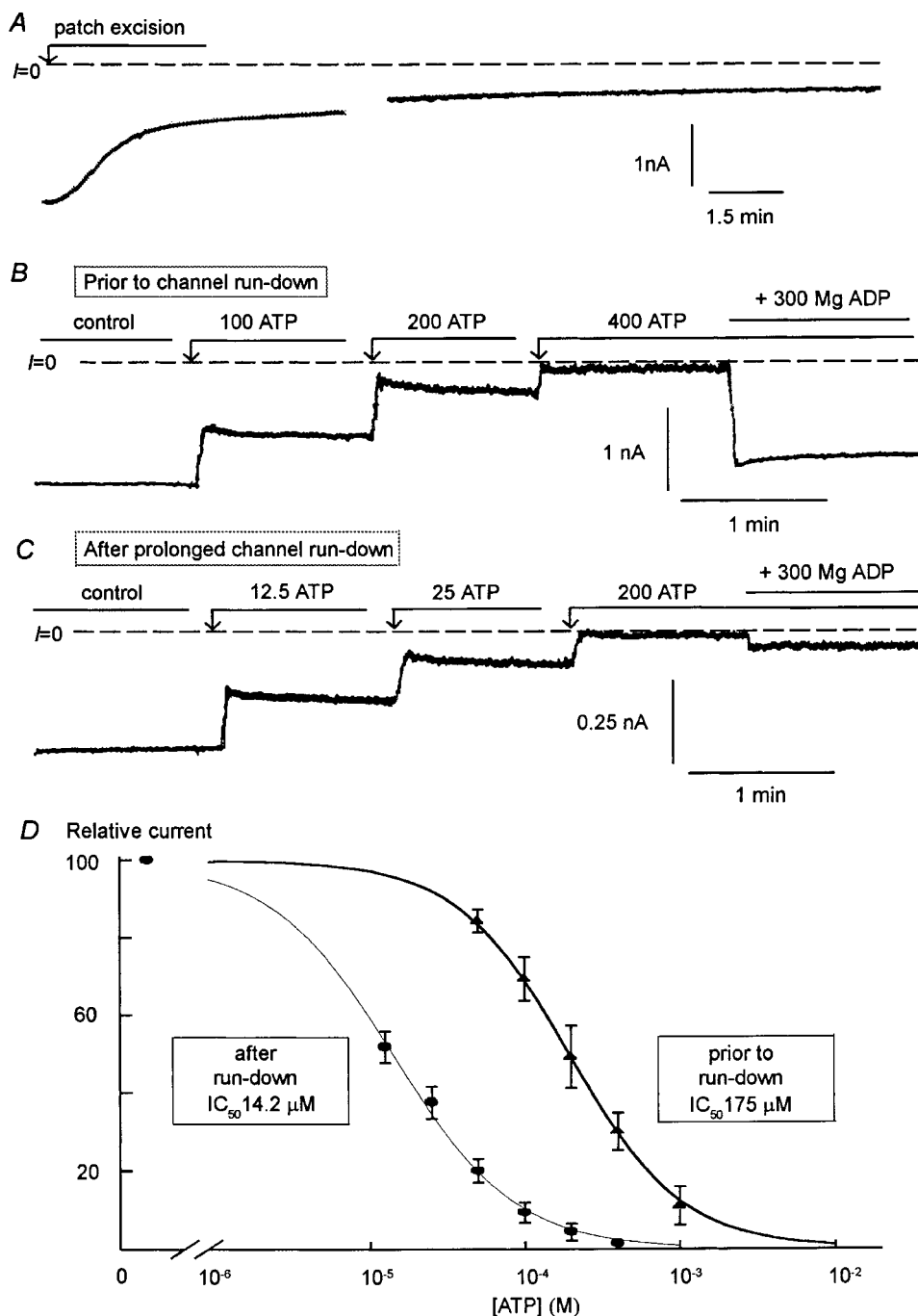


Figure 1. Spontaneous run-down of Kir6.2-GFP+SUR1 channels in excised inside-out patches. (A) Time course of run-down after patch excision into a MgATP-free solution with a Ca^{2+} concentration of 2×10^{-7} M, demonstrating an initial fast phase followed by a slow phase. The dotted line represents zero current, and downward deflections represent inward currents. Holding potential was -10 mV. (B) ATP sensitivity before run-down. Inward current was progressively suppressed by increasing ATP concentrations. The IC_{50} for channel inhibition by ATP was near 100μ M for this patch. In the presence of 400μ M ATP, addition of 300μ M MgADP caused a robust stimulation of channel activity, indicating functional coupling between Kir6.2G and SUR1. (C) The same inside-out patch after prolonged run-down, showing increased sensitivity to ATP with an IC_{50} near 12.5μ M, and loss of stimulation by MgADP, indicating functional uncoupling of Kir6.2G and SUR1. (D) ATP dose-response curves before (\blacktriangle) and after (\bullet) spontaneous prolonged rundown. Data points are the mean \pm SD of six values from different patches. Current amplitude was normalized to the current measured in the same patch in the absence of nucleotide. Lines are best fits to a Hill equation: $I/I_{max} = 1/(1 + ([ATP]/IC_{50})^n)$, with the IC_{50} values listed and $n = 1.1-1.2$.

the addition of 150μ M MgADP to the bath before patch excision prevented fast rundown (Fig. 2 A). In the two patches that showed fast rundown with MgADP present, MgADP did not reactivate ATP-inhibited channels and evoked channel inhibition per se. As discussed later, this occasional observation may be explained assuming that channel dephosphorylation and uncoupling of Kir6.2 and SUR1 occurred at the time of patch excision. In the majority of patches (86%) in which MgADP prevented channel rundown, MgADP also re-

activated ATP-inhibited channels by decreasing their sensitivity to ATP. The degree of this well-known effect of MgADP (see, for example, Mislér et al., 1986; Findlay, 1987) varied from patch to patch, but fell in two categories. In the first category (Fig. 2 D, left bar), the averaged channel sensitivity (IC_{50}) to ATP was $151 \pm 57 \mu$ M in the presence of MgADP and $29.5 \pm 14 \mu$ M in its absence. In the second group (Fig. 2 D, right bar), the respective values were $380 \pm 50 \mu$ M (Fig. 2 B) and $171 \pm 47 \mu$ M (Fig. 2 C). Furthermore, upon MgADP

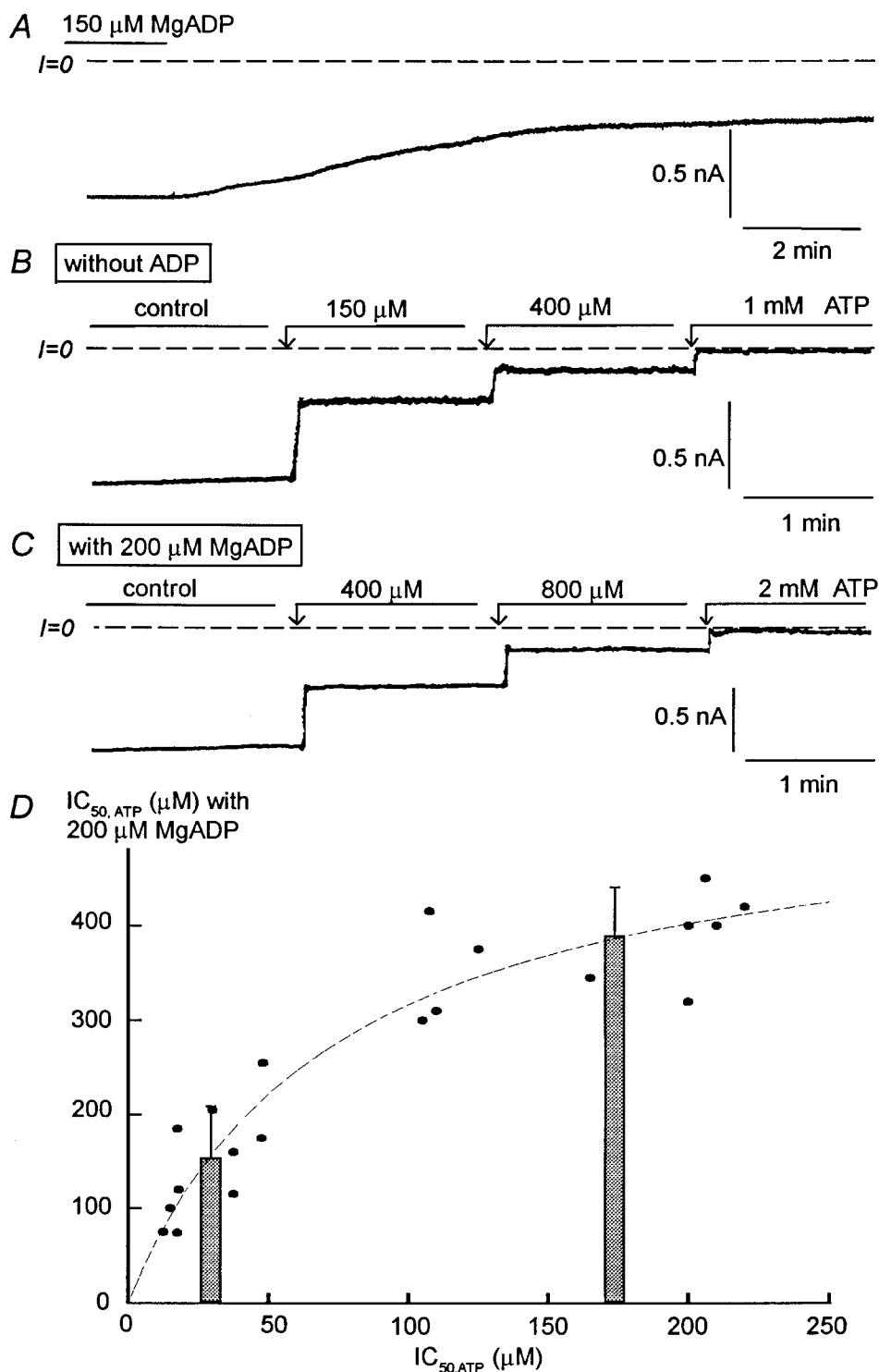


Figure 2. Spontaneous rundown of Kir6.2-GFP+SUR1 channels and the effects of MgADP. (A) Inward current from an inside-out patch excised into bath solution containing 150 μM MgADP, demonstrating absence of fast rundown until MgADP was removed. (B and C) Representative data from inside-out patches showing inward currents with little rundown that are suppressed by increasing ATP concentrations with and without 200 μM MgADP, respectively. In the absence of MgADP (C), the IC_{50} for inhibition by ATP was near 150 μM ; in the presence of MgADP (B), the IC_{50} value was near 400 μM . (D) Correlation between channel ATP sensitivity with and without 200 μM MgADP. Patches were grouped in two categories. In the first category, which exhibited a pronounced fast phase of rundown, the average IC_{50} for inhibition by ATP was 30 ± 14 μM in the absence of MgADP and 152 ± 61 μM with 200 μM MgADP. In the second category, with little or no fast rundown, the average IC_{50} for inhibition by ATP was 171 ± 46 μM in the absence of MgADP and 380 ± 50 μM with 200 μM MgADP. The dotted line was fit using the equation $y = 1/[1 + (k/[ATP])^n]$, which yielded $k = 74$ μM and $n = 0.96$. The latter suggests that one MgADP is sufficient to shift the ATP sensitivity.

removal, the patches in the first group with high sensitivity to ATP exhibited fast rundown ($>30\%$), whereas patches in the second group with low ATP sensitivity exhibited almost no fast rundown ($<10\%$). These two sets of values for ATP sensitivity in the presence and absence of MgADP were used to generate part of the

model presented in the discussion. Finally, the ability of MgADP to prevent fast rundown was only observed if MgADP was present at the time of patch excision (Fig. 2 A). If MgADP was added after fast rundown had begun, it was no longer protective.

Slow rundown. The slow phase of rundown was also

characterized by an increase in ATP sensitivity, with the IC_{50} of all patches averaging $14 \pm 4 \mu\text{M}$ after 1–2 h of slow rundown. Unlike fast rundown, however, the increase in ATP sensitivity was accompanied by an almost complete loss of MgADP-dependent reactivation of ATP-inhibited channels (Fig. 1 C) and of inhibition by sulfonyleureas (data not shown). This loss of MgADP-dependent reactivation (Findlay, 1987; Bokvist et al., 1991; Terzic et al., 1994) and sulfonyleurea-induced inhibition (Gillis et al., 1989) observed during rundown had already been reported for native K_{ATP} channels and suggests functional uncoupling of Kir6.2-GFP and SUR1, since SUR1 confers MgADP and sulfonyleurea sensitivity to Kir6.2+SUR1 channels (Tucker et al., 1997; John et al., 1998).

In summary, we found that spontaneous rundown of Kir6.2-GFP+SUR1 involved two phases. The initial fast phase begun immediately after patch excision was associated with marked increase in ATP sensitivity, probably attributable to the decrease in channel activity (Koster et al., 1999), and could be prevented in most patches by excising the patch into MgADP-containing solution. Right after fast rundown, MgADP still reactivated ATP-inhibited channels. In contrast, the slower phase of rundown, which evoked further decrease in channel activity and increase in ATP sensitivity, led to loss of MgADP-dependent reactivation. The loss of MgADP-dependent reactivation was irreversible and coincided with loss of sulfonyleurea sensitivity, suggesting functional uncoupling of Kir6.2-GFP and SUR1.

Ca²⁺-induced Rundown also Affects ATP Sensitivity and Subunit Coupling

Addition of Ca^{2+} ($10 \mu\text{M}$) to the bath solution blocked K_{ATP} channel activity within seconds, and recovery was minimal after Ca^{2+} removal. For these reasons, Ca^{2+} application has been used as a model to study K_{ATP} channel rundown (e.g., Ohno-Shosaku et al., 1987; Furukawa et al., 1996). Accordingly, we investigated the effects of Ca^{2+} on channel ATP sensitivity and coupling of SUR1 to Kir6.2-GFP. Fig. 3 A shows that addition of Ca^{2+} to the bath solution caused almost complete channel inhibition within seconds, which was poorly reversible upon Ca^{2+} removal. MgATP blocked the residual current, and reactivation of the current was apparent when MgATP was removed after several minutes (Fig. 3 A). AMP-PNP also suppressed the residual current after Ca^{2+} application, but did not reactivate the channels, as was previously shown (Ohno-Shosaku et al., 1987). Thus, reactivation by MgATP after Ca^{2+} -induced rundown involves a phosphorylation process.

The degree of reactivation by MgATP after Ca^{2+} application was dependent on the duration of exposure to Ca^{2+} . After short exposures (1 min or less), channel activity could be completely restored, but, after a 5-min

exposure to Ca^{2+} , reactivation by MgATP never exceeded 10–15% of control activity. After short Ca^{2+} application, the ATP sensitivity of the channels increased (Fig. 3, B and C). In five patches with an initial IC_{50} of $134 \pm 42 \mu\text{M}$ ATP, exposure to $10 \mu\text{M}$ Ca^{2+} for ~ 1 min decreased the average IC_{50} to $12 \pm 4 \mu\text{M}$. Such brief exposures to Ca^{2+} had modest effects on MgADP-dependent reactivation of ATP-inhibited channels, as illustrated in Fig. 3 D, where open circles represent data obtained after Ca^{2+} -induced rundown and closed circles data before Ca^{2+} application (Fig. 2 D).

In contrast, after a 5-min exposure to Ca^{2+} , the reversal of ATP-dependent inhibition evoked by $200 \mu\text{M}$ MgADP decreased from $57 \pm 27\%$ before (Fig. 3 B), to $12 \pm 7\%$ after (C) Ca^{2+} application. Prolonged Ca^{2+} exposure also prevented channel inhibition by sulfonyleureas (data not shown). Thus, short exposure to Ca^{2+} evoked increased channel sensitivity to ATP, while prolonged Ca^{2+} exposure caused functional uncoupling of Kir6.2-GFP and SUR1, which was irreversible.

Regulation of Kir6.2-GFP+SUR1 by PIP₂

According to the PIP_2 hypothesis (Hilgemann and Ball, 1996), Ca^{2+} induces K_{ATP} channel rundown by activating Ca^{2+} -sensitive phospholipase C, which hydrolyzes PIP_2 to levels that no longer stimulate channel activity. Consistent with this hypothesis, Fig. 4 A shows that PIP_2 , like MgATP (Fig. 3 A), reactivated channels that were first inhibited by Ca^{2+} . However, reactivation by PIP_2 was dependent on the duration of Ca^{2+} exposure. The addition of $50 \mu\text{M}$ PIP_2 to the bath evoked within 1 min a robust increase in channel activity when Ca^{2+} was applied for 1 min or less (Fig. 4 A), but after prolonged Ca^{2+} exposure (>5 min), the channel activity remaining after Ca^{2+} removal was insensitive to PIP_2 (Fig. 4 B). Spontaneous rundown had similar effects on channel regulation by PIP_2 . After fast spontaneous rundown, PIP_2 could stimulate channel activity, but, after slow spontaneous rundown, PIP_2 was ineffective (data not shown).

To further characterize the effects of PIP_2 , we examined the channel sensitivity to ATP after Ca^{2+} -induced rundown, and then after PIP_2 -mediated channel reactivation (Fig. 4, C–E). After brief Ca^{2+} -induced rundown, addition of PIP_2 reactivated the channels within 1 min and shifted the IC_{50} from 14 ± 4 to $185 \pm 54 \mu\text{M}$. After 3–5 min, PIP_2 further decreased the ATP sensitivity to $476 \pm 92 \mu\text{M}$ ($n = 5$) without changing the current amplitude. These results are consistent with previous reports by Shyng and Nichols (1998) and Baukowitz et al. (1998). After prolonged Ca^{2+} -induced rundown (>5 min), channel activity did not increase significantly with PIP_2 (Fig. 4 B), but prolonged exposure (~ 10 min) still caused a dramatic decrease in channel sensitivity to ATP, with the IC_{50} increasing to 5.4 mM in three patches (Fig. 4 E, \blacktriangle). In the absence of any signif-

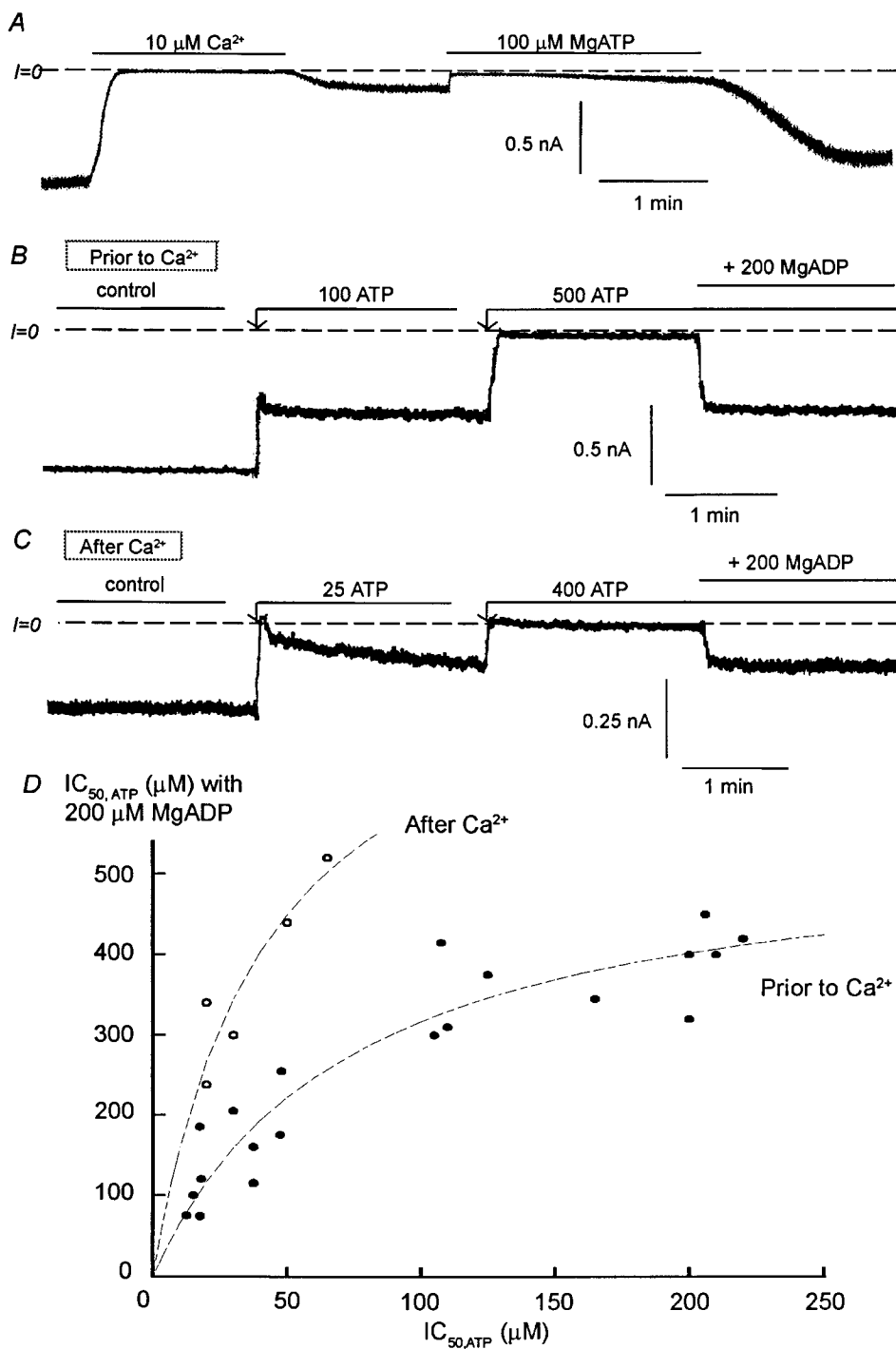


Figure 3. Ca^{2+} -induced rundown and reactivation in Kir6.2-GFP+SUR1 channels. (A) Brief application of 10 μM Ca^{2+} , for ~ 1 min, to the cytoplasmic surface of an excised inside-out patch rapidly inhibited channel activity, which recovered only minimally after removal of Ca^{2+} . Subsequent application of 100 μM MgATP inhibited the residual current, but reactivated the channels, as evidenced by the increase in current after MgATP removal. The currents are inward, with the zero current level indicated by the dashed line. (B and C) ATP sensitivity in a representative inside-out patch before (B) and after (C) exposure to 10 μM Ca^{2+} for 1 min. Inward current was progressively suppressed by increasing [ATP] as indicated. The IC_{50} after Ca^{2+} exposure decreased from near 100 to ~ 15 μM . Stimulation by 200 μM MgADP remained potent after short exposure to Ca^{2+} causing rundown. (D) Plot of channel ATP sensitivity with and without 200 μM MgADP, before (\bullet) and after (\circ) short exposure (< 1 min) to Ca^{2+} . Brief exposure to Ca^{2+} steepens the relationship as the ATP sensitivity increases without major decrease in MgADP-dependent reactivation. After long exposure to Ca^{2+} , MgADP no longer reactivates the channel and the curve dropped below that obtained before Ca^{2+} application (not shown).

icant change in channel activity, this dramatic decrease in channel sensitivity to ATP evoked by PIP_2 after prolonged exposure to Ca^{2+} suggests a direct effect of PIP_2 on ATP binding affinity.

PIP_2 also affected MgADP-dependent reactivation of ATP-inhibited channels. In five experiments, before application of PIP_2 , channel reactivation by MgADP averaged $42 \pm 14\%$ of control (Fig. 4 C). In contrast, after a

5-min exposure to PIP_2 , which dramatically decreased the channel sensitivity to ATP, MgADP-dependent reactivation was suppressed to $8 \pm 4\%$ (Fig. 4 D). Under similar experimental conditions, sulfonyleurea-dependent inhibition was also dramatically suppressed (Koster et al., 1999; Ribalet 1999). In summary, PIP_2 restored channel activity and reversed the increase in ATP sensitivity evoked by Ca^{2+} -induced rundown, but

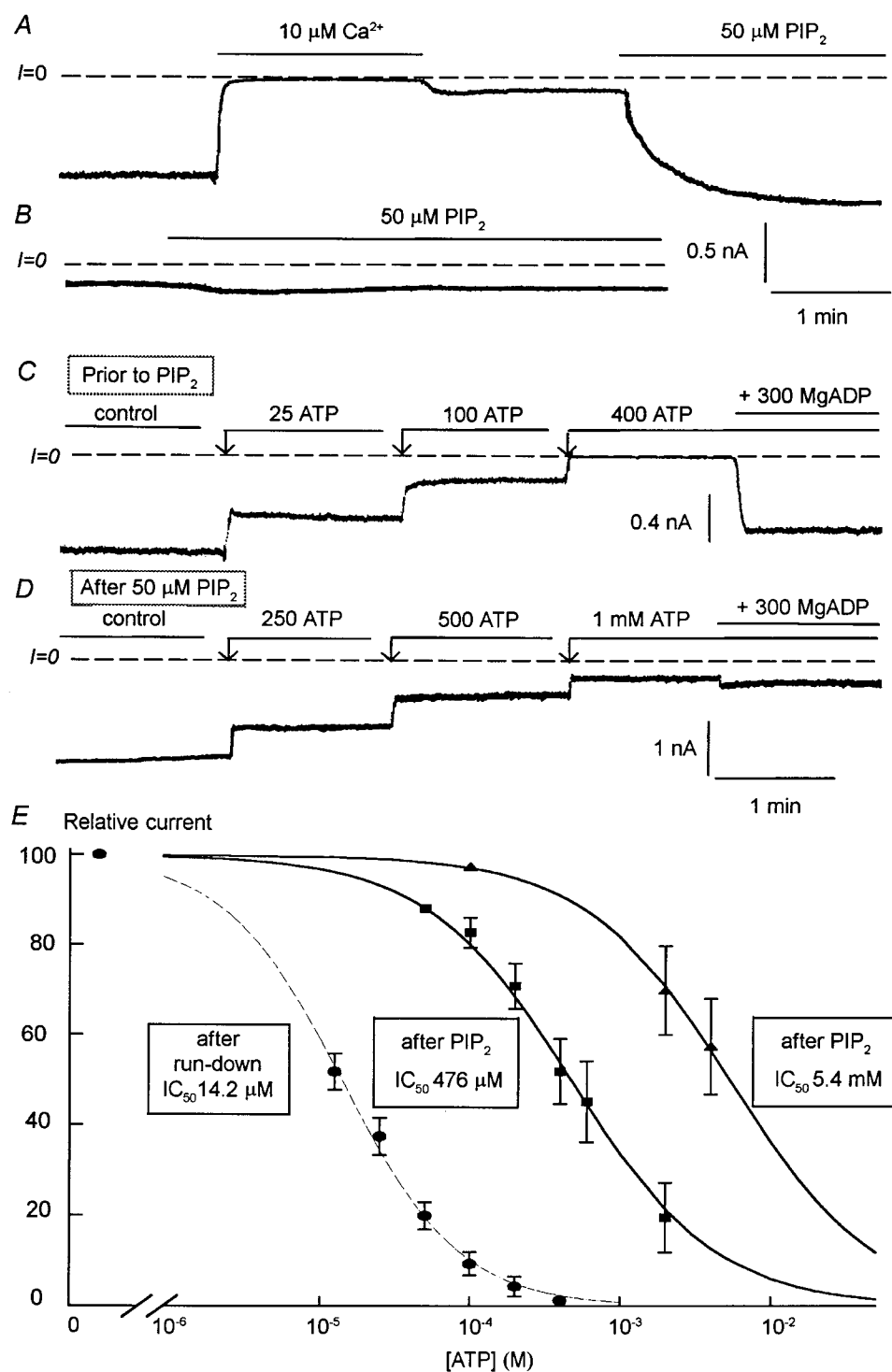


Figure 4. Regulation of Kir6.2-GFP+SUR1 channel sensitivity to ATP and MgADP by PIP_2 . (A and B) Representative inward currents from excised inside-out patches exposed to $50\ \mu\text{M}\ \text{PIP}_2$ after rundown caused by brief (A) or prolonged (B) exposure to $10\ \mu\text{M}\ \text{Ca}^{2+}$. Dashed lines indicate zero current level. PIP_2 led to full recovery of channel activity after brief Ca^{2+} -induced rundown, but minimal recovery after prolonged ($>5\ \text{min}$) exposure to Ca^{2+} . (C and D) ATP sensitivity in representative inside-out patches after fast spontaneous rundown, and before (C) or after (D) exposure to PIP_2 . Inward currents were progressively suppressed by increasing ATP concentrations, with the IC_{50} after PIP_2 increasing from near 25 to $>250\ \mu\text{M}$. Treatment with PIP_2 caused loss of MgADP-dependent reactivation when $300\ \mu\text{M}\ \text{MgADP}$ was applied in the presence ATP. (E) ATP dose-response curves for inside-out patches after brief Ca^{2+} -induced rundown (●) and reactivation by PIP_2 (■), showing a marked increase in the IC_{50} . Also shown (▲) is the ATP dose-response curve after prolonged Ca^{2+} -induced rundown and subsequent exposure to PIP_2 for $>10\ \text{min}$. Although PIP_2 did not significantly increase current amplitude (B), it markedly increased the IC_{50} to the millimolar range. Data points are the mean \pm SD of three to five values from different patches. Current amplitude was normalized to the current measured in the same patch in the absence of nucleotide. Lines are best fits to a Hill equation, as described in Fig. 1.

also prevented channel regulation by MgADP and sulfonylureas after prolonged applications.

In addition to rendering the channel insensitive to inhibition by ATP and reactivation by MgADP, PIP_2 inhibited the ability of Ca^{2+} to induce rundown. Before PIP_2 exposure, block of channel activity by Ca^{2+} persisted after removal of Ca^{2+} (Figs. 5 A and 3 A). How-

ever, after exposure to PIP_2 , channel activity recovered spontaneously when Ca^{2+} was removed (Fig. 5 B), even after a more than 10-min exposure to Ca^{2+} ($n = 12$). This latter result suggests that Ca^{2+} may directly block K_{ATP} channels. Lastly, PIP_2 exposure almost completely prevented any further rundown of channel activity, which decreased by $<10\%$ within 30 min ($n = 8$). To-

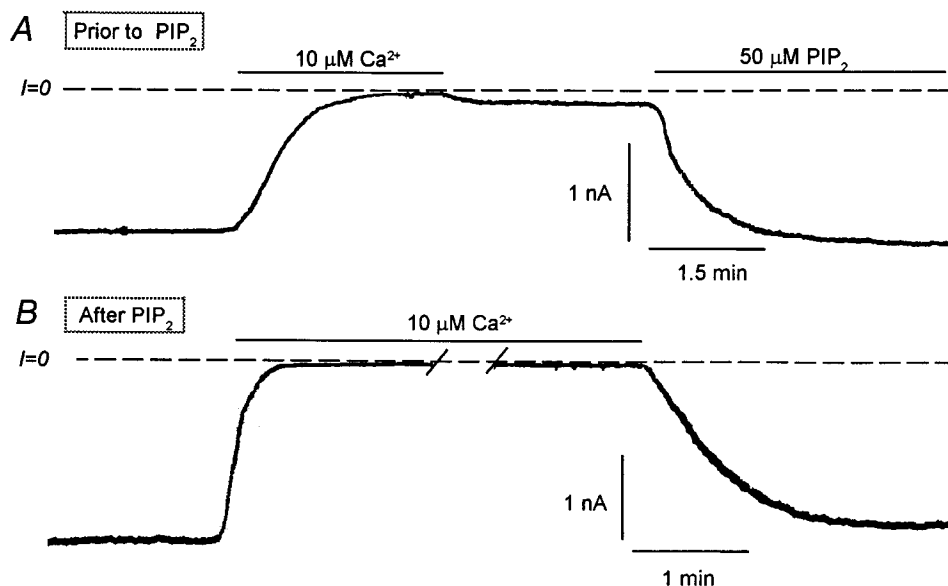


Figure 5. PIP_2 affects Ca^{2+} -induced channel inhibition. (A) Data similar to that in Fig. 4 A, where Ca^{2+} -inhibited channels are reactivated by application of $50 \mu\text{M PIP}_2$. (B) After reactivation by PIP_2 , subsequent application of $10 \mu\text{M Ca}^{2+}$ for up to 10 min blocked inward current, but did not cause rundown, since the current recovered once Ca^{2+} was removed.

gether, these data indicate that reactivation by PIP_2 does not restore the channel to the same state as before rundown, suggesting that spontaneous rundown of Kir6.2-GFP+SUR1 must involve other factors besides PIP_2 hydrolysis. Experiments were performed to identify these factors.

Regulation of Kir6.2-GFP+SUR1 by Protein Kinases

Given earlier evidence of K_{ATP} channel regulation by protein kinases (Hu et al., 1996; Light et al., 1996; Liu et al., 1996), we examined whether rundown may involve dephosphorylation of the channel or a closely associated protein. Accordingly, we tested the effects of protein kinase inhibitors on channel rundown and regulation by ATP and MgADP. Fig. 6 shows that the non-selective protein kinase inhibitor H-7 modestly accelerated rundown of Kir6.2-GFP+SUR1 channel activity. After addition of $100 \mu\text{M H-7}$, channel activity began to decay after 1–2 min and slowly decreased by $22 \pm 7\%$ after 10 min ($n = 6$) (Fig. 6 A). After removal of H-7, addition of $100 \mu\text{M MgATP}$ completely and reversibly blocked channel activity. Okadaic acid, a phosphatase inhibitor, prevented channel inhibition by $100 \mu\text{M H-7}$ (Fig. 6 A), indicating that active phosphorylation-dephosphorylation cycling is occurring in the excised patches, similar to previous conclusions of Light et al. (1996) for native cardiac K_{ATP} channels.

To identify the protein kinase involved, we studied the effects of selective protein kinase inhibitors. The selective protein kinase A inhibitor PKI had almost no effect (data not shown), but the selective PKC inhibitor peptide 19-36 ($5 \mu\text{M}$) evoked a $13 \pm 8\%$ decrease in channel activity after 10 min ($n = 5$) (Fig. 6 B), similar to H-7. These data are consistent with findings in native K_{ATP} channels reported previously (Ribalet and Eddle-

stone, 1995; Hu et al., 1996; Light et al., 1996; Liu et al., 1996) implicating PKC in K_{ATP} channel regulation.

Effects of protein kinase inhibition on ATP and MgADP sensitivity. To further characterize rundown induced by protein kinase inhibitors, we tested the effects of H-7 and peptide 19-36 on ATP and MgADP sensitivity. In five patches with minimal initial fast rundown, H-7 decreased the IC_{50} for channel inhibition by ATP from 175 ± 34 to $8.5 \pm 5.5 \mu\text{M}$ (Fig. 6 E). These data are consistent with previous results indicating that PKC activation decreased the ATP sensitivity of native K_{ATP} channels (Hu et al., 1996; Light et al., 1996).

With H-7 or peptide 19-36, the increase in ATP sensitivity, which occurred within ~ 5 min, was concomitant with the loss of MgADP-dependent reactivation of ATP-inhibited channels. As shown in Fig. 6 D, the presence of MgADP did not prevent channel inhibition by the PKC inhibitor peptide 19-36. In fact, when MgADP was present, channel inhibition by H-7 or PKC inhibitor peptide 19-36 reached $48 \pm 12\%$ after 7 min ($n = 4$), greatly exceeding the inhibition observed in the absence of MgADP (13–22%) for H-7 and peptide 19-36. This reflected the concomitant loss of MgADP stimulation, unmasking the inhibitory effect of ADP. When MgADP was removed, channel activity increased to level similar to that after PKC inhibitor peptide 19-36 treatment in the absence of MgADP (Fig. 6 D, compare with Fig. 4 B). Even 5–10 min after removal of the protein inhibitor, reapplication of MgADP still caused channel inhibition rather than stimulation (Fig. 6 D), indicating that the loss of MgADP stimulation was poorly reversible. After 7 min in the presence of H-7, reactivation by $300 \mu\text{M MgADP}$ reached only $8 \pm 5\%$ of control (Fig. 6 C). At this time, however, PIP_2 could still fully restore channel activity (Fig. 7 B). Much longer (10–15 min)

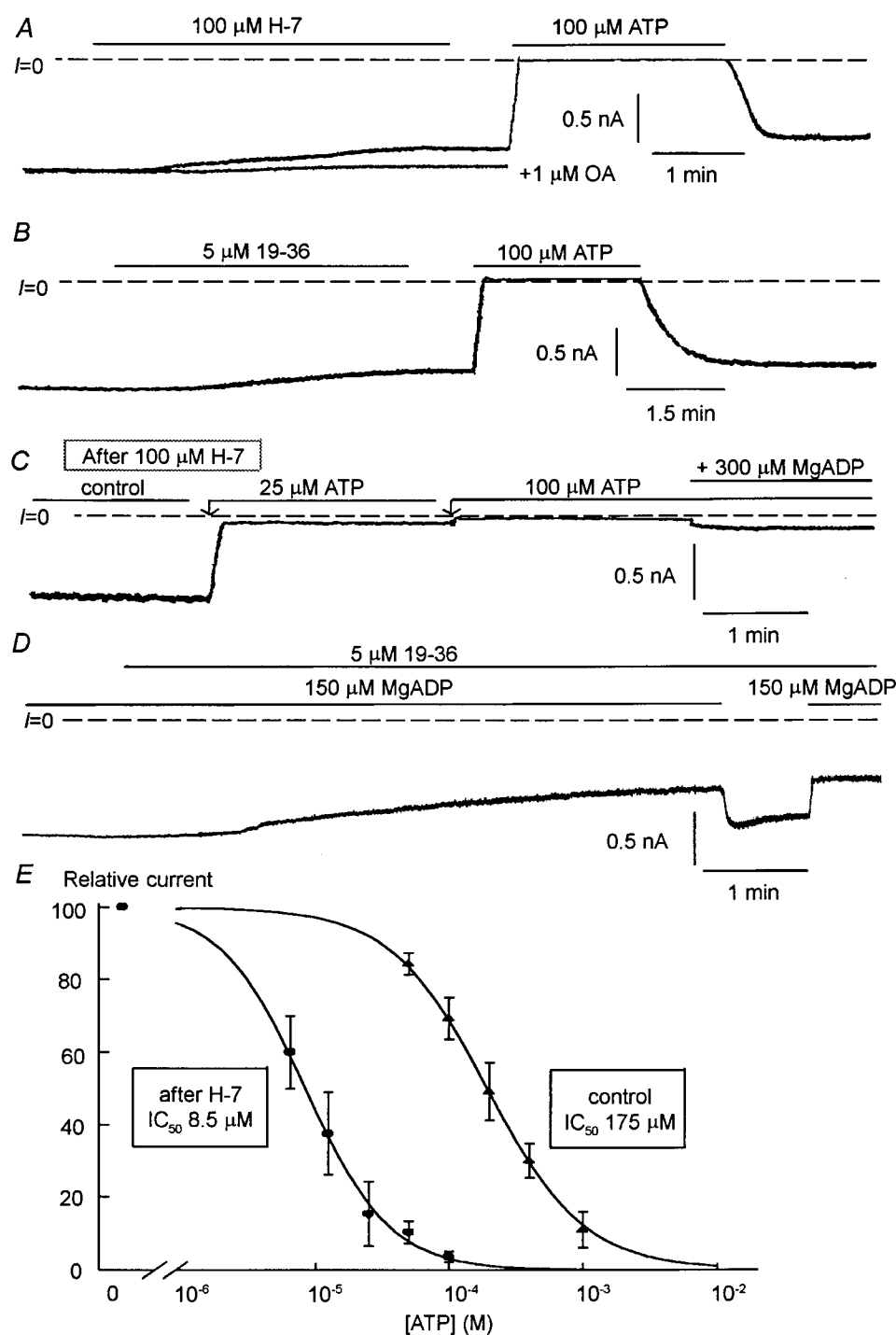


Figure 6. Protein kinase inhibitors affect Kir6.2-GFP+SUR1 channel regulation by ATP and MgADP. (A) Application of 100 μM H-7, a nonselective protein kinase inhibitor, to the cytoplasmic surface of an excised inside-out patch modestly accelerated rundown, which was stabilized when H-7 was removed. After H-7 removal, channel activity was completely suppressed by 100 μM MgATP, but did not recover beyond the rundown level when MgATP was removed, indicating that the protein kinase inhibitor has irreversible effects. The superimposed trace from another patch shows that 1 μM okadaic acid (OA) prevented rundown by H-7. Currents are inward, with zero-current levels indicated by the dashed line. (B) Similar results were obtained using the selective PKC inhibitor peptide 19-36 (5 μM) in place of H-7. (C) ATP sensitivity in a representative inside-out patch after treatment with H-7. Inward current was progressively suppressed by increasing ATP concentrations, with the IC_{50} well below 25 μM . Application of 300 μM MgADP did not significantly stimulate channel activity after H-7, indicating functional uncoupling from SUR1. (D) Representative patch excised into MgADP-containing bath solution to prevent fast rundown. This panel shows that application of the selective PKC inhibitor peptide 19-36 (5 μM) induced rundown and uncoupling from SUR1 even in the presence of MgADP. In this case, inhibition by ADP became manifest due to functional uncoupling of Kir6.2 and SUR1, as evidenced by MgADP removal and reapplication at the end of the trace. (E) ATP dose-response curves for inside-out patches before (▲) and after (●) H-7 induced rundown, showing a marked decrease in

the IC_{50} after H-7. Data points are the mean \pm SD of four to five values from different patches. Current amplitude was normalized to the current measured in the same patch in the absence of nucleotide. Lines are best fits to a Hill equation, as described in Fig. 1.

exposures to protein kinase inhibitors were then necessary to suppress PIP_2 -dependent channel stimulation.

In summary, it was not possible to assess whether, during prolonged spontaneous rundown, the loss of MgADP and of PIP_2 -dependent channel activation involved more than one process. The experiments performed with the protein kinase inhibitors now clearly

indicate that the two phenomena can be temporally dissociated and are therefore different. The demonstration that dephosphorylation-induced rundown causes uncoupling of SUR1 from Kir6.2 also supports the hypothesis that the uncoupling evoked by Ca^{2+} -induced rundown involves a dephosphorylation process very likely mediated by a Ca^{2+} -activated phosphatase.

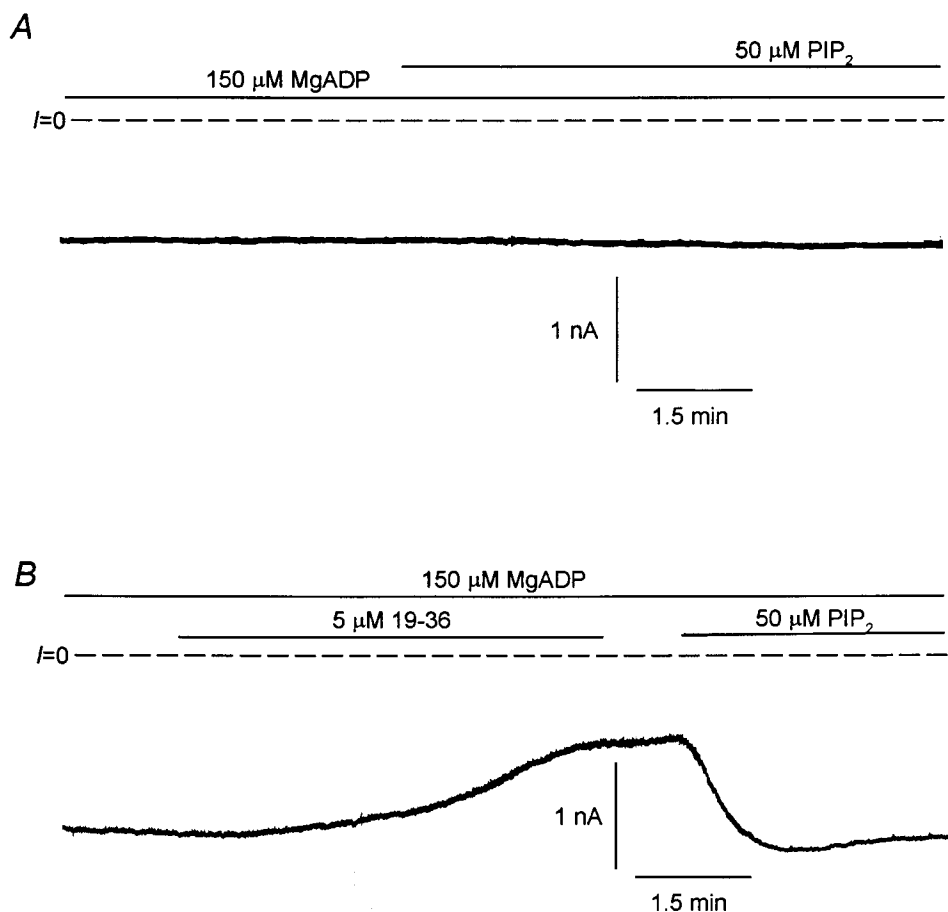


Figure 7. Channel phosphorylation and MgADP control Kir6.2-GFP+SUR1 channel regulation by PIP₂. (A) A representative patch excised into bath solution containing MgADP to prevent the fast phase of spontaneous rundown. Exposure to 50 μM PIP₂ had no effect on channel activity in ATP-free solution. (B) In the presence of MgADP, however, when rundown was induced by application of the selective PKC inhibitor peptide 19-36 (5 μM), application of PIP₂ reactivated the rundown channels. Currents were inward in both traces, with the zero current levels indicated by dashed lines.

Interactions Between PIP₂ and MgADP in Kir6.2-GFP+SUR1 Regulation

The effects of MgADP and PIP₂ are similar in that they both stimulate channel activity and thereby decrease ATP sensitivity. The observation that PIP₂ prevents channel regulation by MgADP (Fig. 4 D) raises the possibility that the two modulators may act competitively to regulate channel activity. To test this possibility, we examined the effects of PIP₂ in the presence of MgADP. As shown in Fig. 7 A, when fast rundown was prevented by MgADP, PIP₂ had no stimulatory effect. In contrast, after rundown induced by H-7 or PCK inhibitor peptide 19-36, PIP₂ had potent stimulatory effects even in the presence of 150–300 μM MgADP (Fig. 7 B). The latter result supports the hypothesis that functional coupling of SUR1 to Kir6.2 is not required for channel stimulation by PIP₂. From these data, we may conclude that PIP₂ and MgADP do indeed compete to activate a common regulatory mechanism. However, another possibility exists (discussed later) whereby MgADP and PIP₂ control independent regulatory mechanisms, each capable of producing maximum channel activation.

Run Down and Reactivation of Homomeric Kir6.2-GFP Channels (in the Absence of SUR1)

To determine the extent to which rundown and reactivation of K_{ATP} channels are properties of the Kir6.2-GFP or the SUR1 subunit, we investigated homomeric Kir6.2-GFP channels, which we have previously shown behave similarly to wild-type Kir6.2 channels (John et al., 1998). In contrast to Kir6.2-GFP+SUR1 channels, homomeric Kir6.2-GFP channels were found to exhibit only a slow phase of rundown when excised into MgATP-free bath solution with low Ca²⁺ (Fig. 8 A). The time constant of decay averaged 780 ± 292 s ($n = 5$).

Effects of Ca²⁺. Similar to Kir6.2-GFP+SUR1 channels, rundown of Kir6.2-GFP channels was markedly accelerated by [Ca²⁺] > 5 μM (Fig. 8 A); 1–2 μM had almost no effect and 20 μM had a maximum inhibitory effect, consistent with the PIP₂ hydrolysis mechanism postulated for native K_{ATP} channels (Hilgemann and Ball, 1996). Also, as with Kir6.2-GFP+SUR1 channels, recovery of channel activity after Ca²⁺ removal varied from patch to patch and was related to the time of exposure to Ca²⁺. After brief exposure to Ca²⁺ (<1 min), channel activity partially recovered, while exposure to

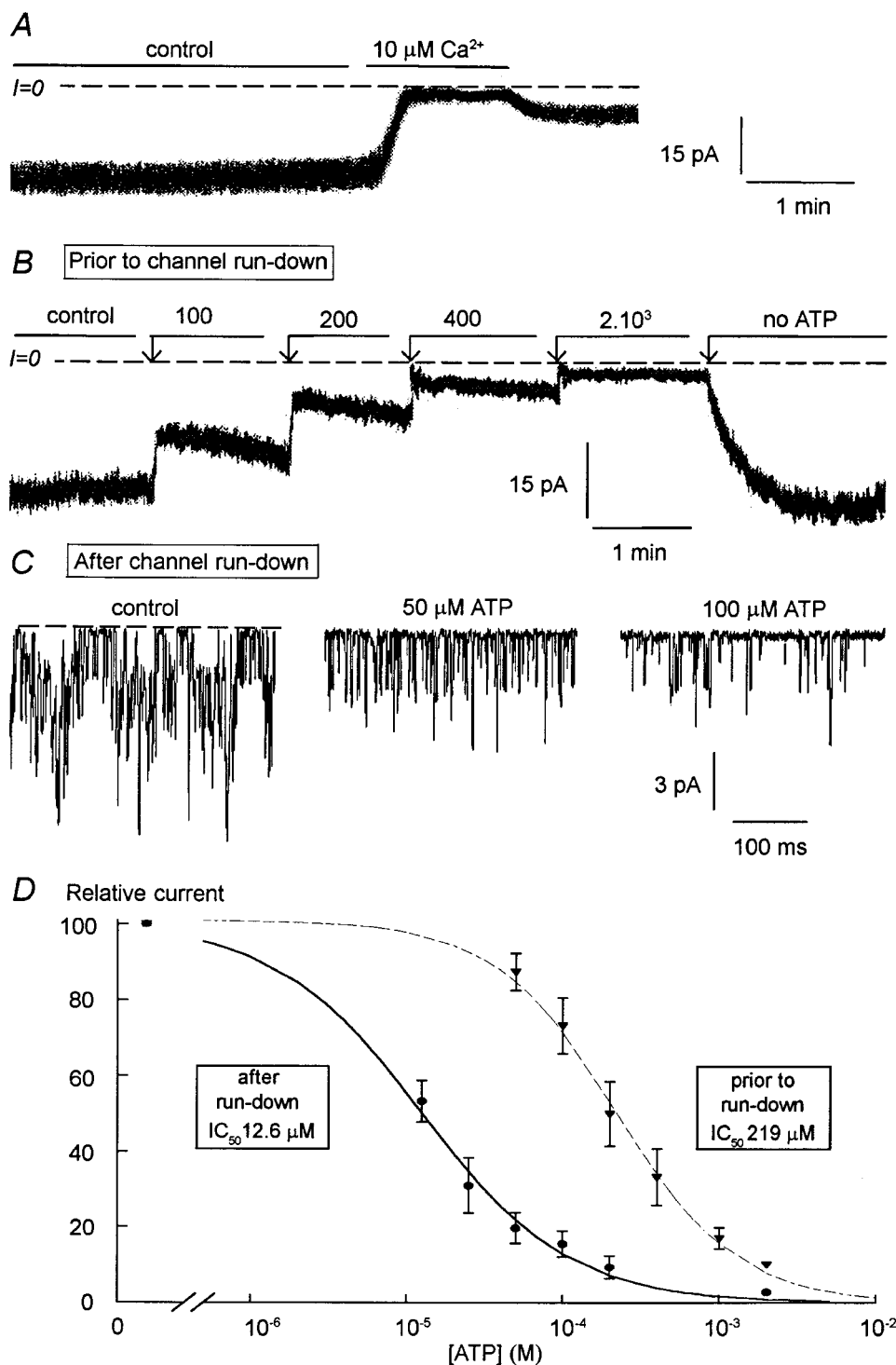


Figure 8. Ca^{2+} -induced rundown is an intrinsic property of the Kir6.2 subunit of the K_{ATP} channel. (A) Representative currents from an excised inside-out patch containing homomeric Kir6.2-GFP channels. Application of 10 $\mu\text{M Ca}^{2+}$ induced rapid channel block, which persisted after Ca^{2+} removal, indicating rundown. (B and C) ATP sensitivity in representative inside-out patches before (B) and after (C) exposure to Ca^{2+} . Inward current was progressively suppressed by increasing ATP concentrations, with the IC_{50} after Ca^{2+} -induced rundown decreasing from near 200 to <50 μM . (E) ATP dose-response curves for inside-out patches before (▼) after (●) Ca^{2+} -induced rundown, showing a marked decrease in the IC_{50} . Data points are the mean \pm SD of three to four values from different patches. Channel activity was normalized to the activity measured in the same patch in the absence of nucleotide. Lines are best fits to a Hill equation, as described in Fig. 1.

Ca^{2+} for >5 min resulted in complete loss of channel activity.

We measured the ATP sensitivity of Kir6.2-GFP channels immediately after patch excision (before rundown) and after Ca^{2+} -induced rundown. Immediately after patch excision, the sensitivity to ATP was low with an IC_{50} of 219 ± 43 μM (Fig. 8, B and D). After a 5-min

exposure to 10 $\mu\text{M Ca}^{2+}$, there was little channel reactivation upon Ca^{2+} removal, and the IC_{50} for ATP decreased to 12.6 ± 2.1 μM (Fig. 8, C and D). We therefore conclude that Ca^{2+} -induced rundown and its effect on ATP sensitivity are, at least in part, an intrinsic property of the Kir6.2 channel subunit.

Effects of PIP_2 . After a brief Ca^{2+} -induced rundown,

PIP₂ reactivated Kir6.2-GFP channels (Fig. 9 A). In addition, similar to Kir6.2-GFP+SUR1 channels, PIP₂ markedly decreased channel sensitivity to ATP, decreasing the IC₅₀ after Ca²⁺-induced rundown from 12.6 to 465 μ M (Fig. 9 B).

Effects of protein kinase inhibition. As with Kir6.2-GFP+SUR1, H-7 and peptide 19-36 caused slow inhibition of Kir6.2-GFP (Fig. 9 C). In five experiments, inhi-

bition averaged $19 \pm 8\%$ of control after 10 min. Channel activity partially recovered after addition and withdrawal of MgATP (Fig. 9 C). These results indicate that protein kinases have a direct stimulatory effect on Kir6.2-GFP, in addition to facilitating coupling between Kir6.2-GFP and SUR1. However, in contrast to Kir6.2-GFP+SUR1 channels, the sensitivity of Kir6.2-GFP channels to ATP was not affected by protein kinase C

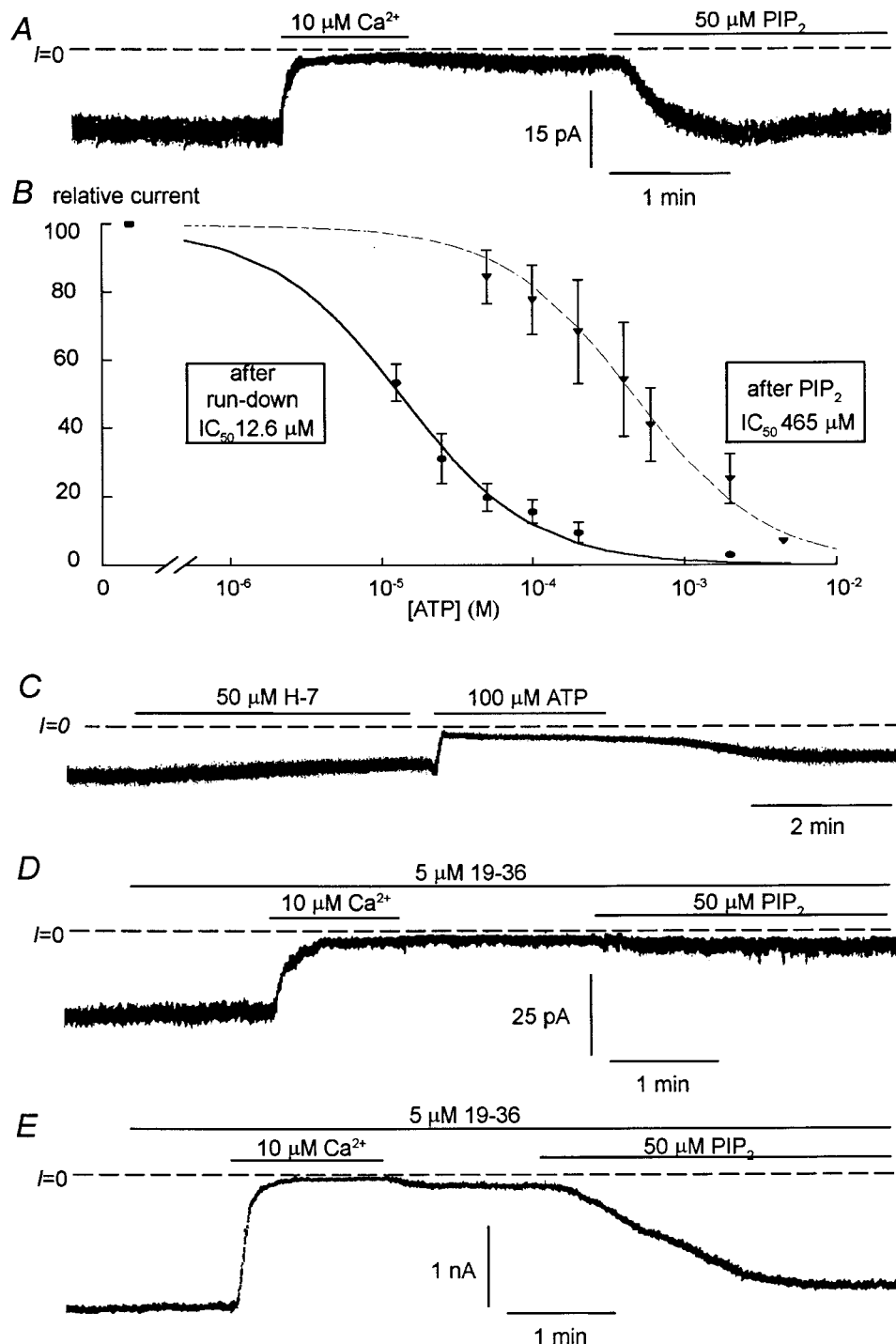


Figure 9. Reactivation of homomeric Kir6.2-GFP channels by PIP₂ after rundown induced by Ca²⁺ and protein kinase inhibitors. (A) Representative excised inside-out patch showing channel reactivation by PIP₂ after rundown due to brief Ca²⁺ application. Currents are inward, with the zero current level indicated by the dashed line. (B) ATP dose-response curves for inside-out patches after Ca²⁺-induced rundown before (●) and after (▼) reactivation by PIP₂, showing a marked increase in the IC₅₀. Data points are the mean \pm SD of three to four values from different patches. Channel activity was normalized to the activity measured in the same patch in the absence of nucleotide. Lines are best fits to a Hill equation, as described in Fig. 1. (C) Currents from a representative inside-out patch demonstrating that nonselective protein kinase inhibition with 50 μ M H-7 accelerated rundown of Kir6.2-GFP channels, similar to Kir6.2-GFP+SUR1 channels. (D and E) Currents from representative inside-out patches, in which rundown was induced by the combined effects of the selective protein kinase inhibitor, peptide 19-36 (5 μ M), and brief application of Ca²⁺ (10 μ M). PIP₂ failed to significantly reactivate Kir6.2-GFP channels (D), but was effective at reactivating Kir6.2-GFP+SUR1 channels (E).

inhibitors even though their activity decreased, possibly because of the nonlinear relationship between P_o and ATP sensitivity (see discussion).

We next compared the ability of PIP_2 to reactivate Kir6.2-GFP channels and Kir6.2-GFP+SUR1 channels after combined protein kinase inhibition and brief Ca^{2+} -induced rundown. With Kir6.2-GFP alone, short exposures to protein kinase inhibitors for 2–3 min followed by brief Ca^{2+} exposure were sufficient to prevent channel reactivation by PIP_2 (Fig. 9 D). In contrast, Kir6.2-GFP+SUR1 channels subjected to the same protocol were readily reactivated by PIP_2 (Fig. 9 E), and prolonged exposure to protein kinase inhibition was required to finally disrupt PIP_2 regulation. Thus, the heteromultimeric channel is less sensitive to treatments that cause slow rundown, suggesting that SUR1 may protect Kir6.2 from dephosphorylation.

Differential Effects of MgATP and PIP_2 on Kir6.2-GFP Single Channel Kinetics

To search for additional evidence of dual regulation of Kir6.2-GFP channels by protein kinases and PIP_2 , we examined the effects of channel reactivation by MgATP and PIP_2 on single Kir6.2-GFP channel kinetics. The rationale is that reactivation by MgATP should facilitate both protein kinase-mediated phosphorylation and PIP_2 resynthesis, and may therefore have different effects than PIP_2 alone. We were unable to analyze Kir6.2-GFP+SUR1 single-channel activity since patches contained too many channels, and, even with Kir6.2-GFP channels, patches typically had more than one channel. Therefore, we restricted our study to the analysis of open-time distributions after Ca^{2+} -induced rundown left only a few active Kir6.2-GFP channels in the patch.

Fig. 10 shows representative single-channel traces and open-time distributions before and after application of either MgATP (Fig. 10, A and B) or PIP_2 (C and D) to reactivate the channels. The open-time histogram obtained before and after MgATP was well fitted by two exponentials. Before MgATP, the time constants of the two exponentials were $\tau_2 = 0.6 \pm 0.2$ ms and $\tau_2 = 2.7 \pm 0.6$ ms and, after MgATP, the time constants were $\tau_2 = 1 \pm 0.3$ ms and $\tau_2 = 3 \pm 0.3$ ms ($n = 4$) (Fig. 10 E). According to the data shown in Fig. 10, B and E, the rebound in channel activity evoked by MgATP was primarily due to a 5.6 ± 1.8 -fold increase in the frequency of short open times. In contrast, the frequency of the burst of openings increased only by 1.2 ± 0.5 -fold.

Similarly, after PIP_2 , the open-time histogram was well fit by two exponentials with time constants of $\tau_2 = 0.8 \pm 0.3$ ms and $\tau_2 = 3.7 \pm 1.1$ ms. After PIP_2 application, the frequency of the burst of openings increased by 5.4 ± 2.3 -fold, while that of short openings increased only moderately by 1.3 ± 0.6 -fold (Fig. 10, C–E). Thus, PIP_2 appeared to facilitate channel transition to a pro-

longed active state, while MgATP primarily facilitated transition to a short open state. The different kinetic effects of MgATP and PIP_2 on Kir6.2-GFP channels' open-time distributions are consistent with two MgATP-dependent processes, mediated by protein kinase and PIP_2 , and they suggest that the major control sites are located on the pore-forming Kir6.2 subunit.

DISCUSSION

The major conclusion of this study is that rundown of K_{ATP} channels involves multiple factors, including MgADP, PIP_2 , and protein kinases. This conclusion is consistent with previous studies describing regulation of native and cloned K_{ATP} channels by protein kinases (Ribalet and Eddlestone, 1995; Hu et al., 1996; Light et al., 1996; Liu et al., 1996), PIP_2 (Furukawa et al., 1996; Hilgemann and Ball, 1996; Fan and Makielski, 1997, 1999; Baukrowitz et al., 1998; Shyng and Nichols, 1998), and nucleotide diphosphates (Findlay, 1987; Bokvist et al., 1991; Terzic et al., 1994). Our findings can be summarized as follows. (a) Spontaneous rundown of Kir6.2-GFP+SUR1 involves a fast phase lasting close to 1 min that caused an increase in ATP sensitivity and that is prevented by MgADP. A slower phase then followed, lasting over tens of minutes to hours that caused a loss of sensitivity to MgADP, sulfonylureas, and PIP_2 . (b) Brief exposure to Ca^{2+} (<1 min) increased only the ATP sensitivity, while prolonged exposure (>5 min) suppressed the channel sensitivity to MgADP, sulfonylureas, and PIP_2 . (c) Protein kinase inhibitors had similar effects, but the loss of MgADP-dependent reactivation preceded the loss of PIP_2 -dependent stimulation. (d) Kir6.2-GFP alone exhibited only a slow phase of rundown, suggesting that fast rundown involves SUR1 and slow rundown is a property of Kir6.2.

The Fast Phase of Rundown: The Role of MgADP

The fast phase of spontaneous rundown is specific to Kir6.2-GFP+SUR1, and therefore involves SUR1. Since MgADP in the bath prevented fast rundown (Fig. 2 A), we postulate that, in the absence of bath-applied MgADP, dissociation of MgADP from SUR1, with consequent loss of MgADP-dependent stimulation, is responsible for fast rundown. Previous studies consistent with this hypothesis have shown that the rate of closure of phosphorylated channels (~ 1 min) upon removal of nucleotide diphosphates (Terzic et al., 1994) is similar to that of fast rundown (Fig. 1 A).

Fast rundown is characterized by an increase in ATP sensitivity. Such an increase in ATP sensitivity results, at least in part, from a decrease in channel activity since a correlation has been demonstrated between P_o and the channel sensitivity to ATP. Thus, decreasing the mean open time would shift the equilibrium towards the

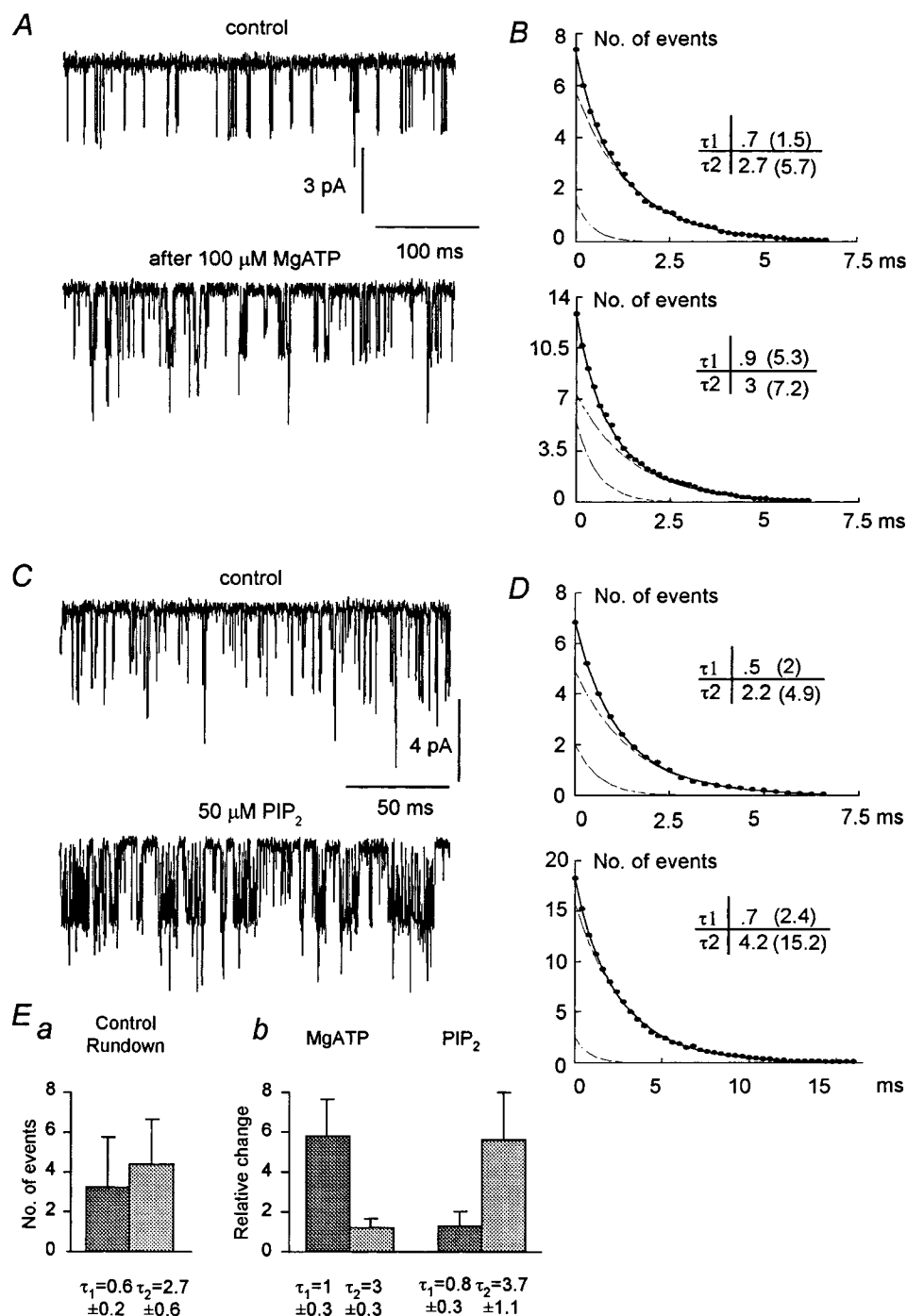


Figure 10. Different effects of reactivation by MgATP and PIP₂ on Kir6.2-GFP single channel kinetics. (A) Representative single channel current recordings from an excised inside-out patch at a holding potential of -25 mV after prolonged spontaneous rundown left only two channels in the patch. Recordings were obtained before (top) and after (bottom) application of $100 \mu\text{M}$ MgATP to reactivate the channels. Downward deflections represent inward currents. (B) Open-time histograms corresponding to the single channel currents in A, fit with two exponentials (dotted lines). (Top) Before MgATP: $\tau_{\text{fast}} = 0.7$ ms and $\tau_{\text{slow}} = 2.7$ ms; (bottom) after MgATP: $\tau_{\text{fast}} = 0.9$ ms and $\tau_{\text{slow}} = 3$ ms. (E, a) MgATP did not significantly affect the two time constants. The tables in B list the amplitudes of the two exponential components in arbitrary units. MgATP increased the number of fast openings by 3.5-fold and the number of long openings by 1.25-fold. (C and D) Corresponding single channel traces and open time histograms from another inside-out patch after prolonged rundown, before (top) and after (bottom) reactivation by $50 \mu\text{M}$ PIP₂. In contrast to MgATP, PIP₂ selectively increased the number of long burst of openings (threefold), while increasing only very slightly the number of short openings (1.2-fold). In addition, PIP₂ increased the duration of the bursts of openings ($\tau_{\text{slow}} = 2.2$ and 4.2 ms before and after PIP₂, respectively). (E, b) Graphs showing averaged τ 's and increased number of events in three patches, after MgATP (left) and PIP₂ (right).

closed state to which ATP binds preferentially, thereby increasing the apparent affinity and sensitivity of the channel to inhibition by ATP (Shyng et al., 1997; Trapp et al., 1998; Tucker et al., 1998; Babenko et al., 1999; Fan and Makielski 1999). Accordingly, the decrease in channel activity and the accompanying increase in ATP sensitivity observed during fast rundown may be due to dissociation of the adenine nucleotide and loss of MgADP-dependent stimulation. As a corollary, the high ATP sensitivity of Kir6.2+SUR1 and native K_{ATP} chan-

nels ($\text{IC}_{50} \cong 15 \mu\text{M}$) reported in many excised patch-clamp studies may result from fast rundown and loss of MgADP-dependent stimulation. This hypothesis is supported by the present data as well as previous reports demonstrating the effect of MgADP on ATP sensitivity, indicating that the physiological ATP sensitivity of Kir6.2+SUR1 and native K_{ATP} channels in the presence of intracellular MgADP may lie between 100 and 400 μM . This "low" ATP sensitivity would be compatible with the role of ATP-sensitive channels as metabolic

sensors designed to respond to physiologically relevant variations in cellular ATP levels.

The Slow Phase of Rundown: Role of PIP₂, Phosphorylation, and SUR1

In contrast to fast rundown, slow rundown was observed with Kir6.2-GFP+SUR1, as well as Kir6.2-GFP alone. Thus, the slow phase is, at least in part, a characteristic of Kir6.2-GFP. Moreover, slow rundown of Kir6.2-GFP alone had at least two components, since brief Ca²⁺ application left the channels reactivatable by MgATP and PIP₂, whereas prolonged Ca²⁺ application did not. Lastly, slow rundown of Kir6.2-GFP+SUR1 exhibits a third feature involving functional uncoupling of Kir6.2 and SUR1, since MgADP-dependent reactivation of ATP-inhibited channels was gradually lost during slow rundown of the heteromeric channel.

Role of PIP₂. With Kir6.2-GFP alone, brief exposure to Ca²⁺ facilitated spontaneous slow rundown by accelerating channel closure and increasing ATP sensitivity. Direct application of PIP₂ or MgATP reversed these effects (Fig. 9). These results are consistent with recent studies of PIP₂ on native and cloned K_{ATP} channels (Furukawa et al., 1996; Hilgemann and Ball, 1996; Fan and Makielski, 1997, 1999; Baukrowitz et al., 1998; Shyng and Nichols, 1998) and suggest that one component of spontaneous slow rundown of Kir6.2-GFP channels involves PIP₂ hydrolysis. In accordance with this hypothesis, PIP₂ hydrolysis ought to be a slow process that requires hours. This may indeed very well be the case at submicromolar free Ca²⁺ concentrations, since our data showed that prolonged channel inhibition by Ca²⁺ was fully reversible when membrane patches had been previously exposed to excess exogenous PIP₂ (Fig. 5 B).

The decrease in ATP sensitivity induced by exogenous PIP₂ could very well be explained by the increase in channel activity evoked by PIP₂ assuming that ATP binds preferentially to a closed state of the channel (Shyng and Nichols, 1998) and that PIP₂ shifts the equilibrium away from the closed state, thereby decreasing the apparent affinity and sensitivity of the channel to inhibition by ATP. According to this hypothesis, when P_o is near-maximal, small absolute changes (e.g., from 0.90 to 0.99), which are experimentally undetectable, could have major effects on ATP sensitivity. However, after slow spontaneous rundown or prolonged exposure to Ca²⁺, channel activity is at a low level and application of PIP₂ does not significantly increase the activity of these rundown channels, yet markedly decreases their ATP sensitivity. This data is not consistent with the former mechanism, and indicates a direct effect of PIP₂ on ATP binding affinity (Fan and Makielski, 1999). Therefore, we conclude that PIP₂ decreases the ATP sensitivity by affecting ATP binding affinity as well as channel gating.

Role of phosphorylation. Protein kinase inhibition facilitates the slow rundown process of Kir6.2-GFP and Kir6.2-GFP+SUR1 channels, preventing reactivation of both channels by PIP₂ and changing the ATP and MgADP sensitivity of the latter channels. These results suggest that a second component of slow rundown is related to protein dephosphorylation, which is consistent with previous studies investigating the effects of protein kinases on K_{ATP} channel function. These studies did not specifically address rundown, but consistently showed that PKC desensitized K_{ATP} channels to ATP (Ribalet and Eddlestone, 1995; Hu et al., 1996; Light et al., 1996; Liu et al., 1996). The increase in ATP sensitivity observed with Kir6.2-GFP+SUR1 channels during protein kinase inhibitor-induced rundown may be attributed in part to a decrease in channel activity due to loss of MgADP and/or PIP₂ stimulation. However, the decrease in channel activity was small (Fig. 6, A and B) compared, for instance, with that measured during fast rundown, and may not account fully for the substantial increase in ATP sensitivity observed. This apparent discrepancy may be related to the nonlinear relationship between P_o in the absence of ATP and channel sensitivity to ATP (Trapp et al., 1998; Enkvetchakul et al., 2000). Interestingly, with Kir6.2-GFP alone, the small decrease in channel activity induced by PKC inhibitors did not cause a significant increase in ATP sensitivity. However, changes in P_o are most effective at altering ATP sensitivity when P_o is near maximal (~ 0.9), as may be the case with Kir6.2-GFP+SUR1. The P_o of Kir6.2, on the other hand, is considerably lower (< 0.5) (Tucker et al., 1997; John et al., 1998), and small changes in this P_o evoked by protein kinase inhibition are thus expected to have little effect on ATP sensitivity. These findings indicate that changes in ATP sensitivity associated with PKC inhibition are mediated primarily by changes in P_o . Alternatively, protein dephosphorylation may also directly decrease the affinity of Kir6.2 for ATP.

Recent studies with Kir6.2 mutants have suggested two types of interactions between ATP and Kir6.2, which involve two distinct regions of the pore-forming protein (Drain et al., 1998; Tucker et al., 1998). Mutations in one region, just outside the M2 transmembrane domain (around amino acids 170–180) cause a decrease in ATP sensitivity together with an increase in P_o . It has been suggested that this region comprises a gate, which interacts with ATP to close the channel. Mutation-induced weakening in this interaction would cause an increase in P_o and an apparent decrease in ATP sensitivity. Mutations in a second region, farther away from the M2 domain (amino acids 334 and 337, which may also include K185), also decrease the channel sensitivity to ATP, but without change in P_o . It has been postulated that this region is part of the ATP binding site. In this

case, the mutations induce a decrease in ATP sensitivity by decreasing the channel affinity for ATP (Drain et al., 1998; Tucker et al., 1998). These observations are consistent with our findings and explain how interaction of PIP₂ and protein kinase with one or the other of these regions may affect the channel sensitivity to ATP by changing P_o or the binding of ATP to the channel. Interestingly, T180, which is part of the region that controls ATP binding, is a consensus site for PKC-dependent phosphorylation. We postulate that the effect of PKC inhibitors on ATP sensitivity, which we present here, may involve dephosphorylation of T180.

We cannot totally exclude that the decrease in channel activity evoked by protein kinase inhibitors may be due to nonspecific inhibition of phosphatidylinositol-kinase causing decreased PIP₂ synthesis. However, channel inhibition by the specific protein kinase inhibitor peptide 19-36, as well as the less specific inhibitor H-7, indicates that protein dephosphorylation is very likely to decrease channel activity during slow rundown. Because H-7 and peptide 19-36 suppress the PIP₂-dependent regulation of Kir6.2-GFP+SUR1 as well as of Kir6.2-GFP alone, we postulate that the inhibitors act by deactivating a PIP₂ regulatory site on Kir6.2, which may include residues R176 and R177 (Fan and Makielski, 1997). It should also be noted that when SUR1 was coexpressed with Kir6.2-GFP, the combined effects of protein kinase inhibitors and Ca²⁺ on PIP₂-dependent regulation were less prominent, suggesting that SUR1 protects Kir6.2-GFP from dephosphorylation. This may contribute to the low P_o of functional homomeric Kir6.2 channels expressed without SUR1.

Uncoupling of Kir6.2-GFP and SUR1. In addition to preventing reactivation by MgATP and PIP₂, dephosphorylation of Kir6.2-GFP+SUR1 channels also caused functional uncoupling of SUR1 from Kir6.2-GFP, since both MgADP-dependent reactivation and sulfonyl-urea inhibition were abolished. Thus, with Kir6.2-GFP+SUR1, the decrease in channel activity associated with slow rundown may have multiple causes, including loss of PIP₂-dependent stimulation as well as uncoupling of Kir6.2-GFP and SUR1.

Dephosphorylation-induced loss of MgADP-dependent reactivation of ATP-inhibited channels may be due to direct uncoupling of SUR1 from Kir6.2-GFP or decreased MgADP binding to SUR's nucleotide-binding fold (NBF), in which case uncoupling would only be apparent or functional. The protein phosphorylation site that regulates coupling between Kir6.2 and SUR1 is probably different from the phosphorylation site that controls PIP₂ regulation, since with Kir6.2-GFP+SUR1 channels subjected to protein kinase inhibitors, the loss of MgADP sensitivity and of PIP₂ stimulation occurred with different time courses (Fig. 7 B). If dephosphorylation-induced uncoupling is assumed to be primarily

functional, it is then conceivable that the phosphorylation site controlling this process would be located on SUR1 rather than on Kir6.2. The hypothesis that phosphorylation of SUR1 favors binding of MgADP and thus functional coupling is supported by the observation that the K1384M mutation in SUR1's second NBF completely suppress MgADP-dependent reactivation of ATP-inhibited channels (Gribble et al., 1997). This lysine is part of a sequence identified as a PKC phosphorylation site (S X K) found in all SURs as well as CFTR second nucleotide-binding fold. Thus, slow rundown of Kir6.2-GFP+SUR1 may involve uncoupling of SUR1 from Kir6.2-GFP, as a result of dephosphorylation of one or both subunits, together with PIP₂ hydrolysis.

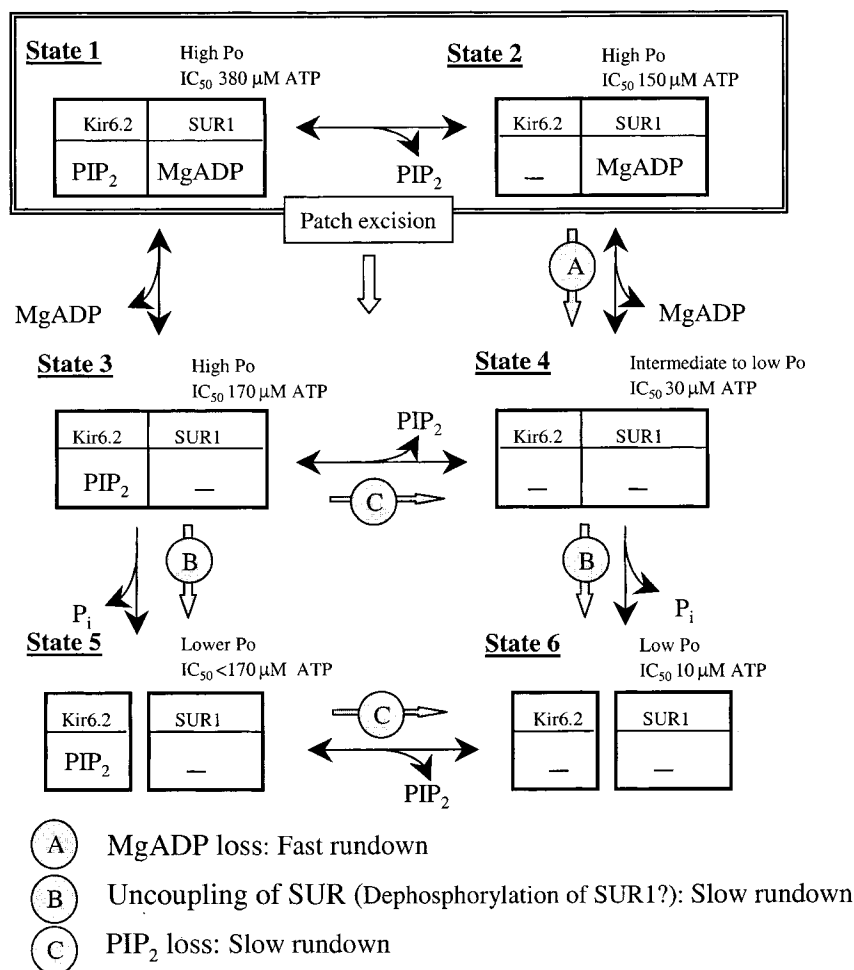
A Model for K_{ATP} Channel Regulation by PIP₂, MgADP, and Phosphorylation

Previous kinetic studies have shown that K_{ATP} channels may exist in different states, with interconversion between states regulated by such factors as nucleotide diphosphates (Terzic et al., 1994; Alekseev et al., 1998) and PIP₂ (Trapp et al., 1998; Fan and Makielski, 1999). Such gating schemes can be extended to account for most of our observations on channel rundown and reactivation. Fig. 11 depicts one possible scheme.

Fig. 11 summarizes how the channel activity can be modulated by PIP₂ and MgADP binding to specific sites on Kir6.2 and SUR1, respectively, and by dephosphorylation. The channel rundown seen experimentally is described by several channel behaviors that occur in parallel or sequentially after patch excision. With phosphorylated channel, fast and slow rundown occur due to loss of MgADP and PIP₂, respectively (pathways A and C). It is assumed that MgADP dissociation is fast, while PIP₂ hydrolysis is slow, and that States 1–3 have similar P_o , which are near maximum, whereas state 4 has a low P_o . The assumption that States 2 and 3 have higher P_o than State 4 is based on the observation that PIP₂ and MgADP stimulate rundown channels. In addition, the P_o of State 1 would not be higher than that of States 2 or 3 because MgADP had no further stimulatory effect on patches that exhibited only slow rundown upon patch excision (transition to State 3) and PIP₂ had no obvious stimulatory effect either when patches were excised in the presence of MgADP. The highly variable degree of fast rundown among excised patches can be explained by assuming differing levels of channels in States 1 and 2 before patch excision. With patches with most channels in State 1 (PIP₂ and MgADP regulated), there would be no fast rundown upon dissociation of MgADP, as PIP₂ remains bound, whereas with patches with most channels in State 2 (MgADP regulated), fast rundown would be prominent as MgADP dissociates.

For slow rundown, one pathway involves PIP₂ hydroly-

A



B

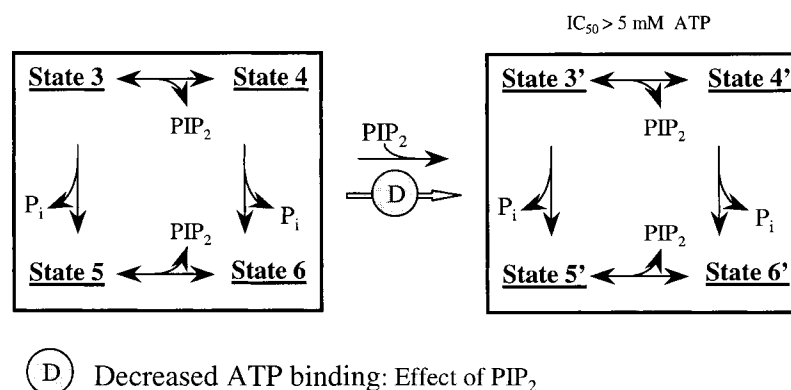


Figure 11. Model for K_{ATP} channel regulation by PIP₂, MgADP, and phosphorylation. In this model, channels are formed by Kir6.2 and SUR1 subunits, which have regulatory sites for PIP₂ and MgADP, respectively. (A) States 1 and 2 represent two physiological states in intact cells, which differ by their PIP₂ regulatory site occupancy. The distribution between these two states is determined by the cellular level of PIP₂. The other four states result from rundown that occurs via alternate pathways (A–C) after patch excitation. States 1–3 have similar high P_o and occupancy of one or both regulatory sites causes maximum channel activation (high P_o). Only State 4, which has both regulatory sites free, exhibits a low P_o . The low P_o of State 4 accounts, at least in part, for its high ATP sensitivity, and the high P_o in the other states accounts for their low ATP sensitivity (see text for explanation). After dissociation of MgADP (pathway A), the transition from State 1 to State 3 has little effect on channel activity, since both states have similar P_o , only transition from State 2 to 4 leads to fast rundown. Transition from State 1 to 3 and 4 leads to slow rundown due to loss of PIP₂ (pathway C). In this case, the loss of PIP₂ (State 3 to 4) is much slower than the loss of MgADP (State 1 to 3) and is, therefore, the rate-limiting step. Another form of rundown occurs as a result of dephosphorylation (pathway B). Dephosphorylation induces loss of MgADP-dependent reactivation of ATP-inhibited channels and is taken as evidence for functional uncoupling of SUR1 and Kir6.2. In the model, this is represented by the transitions from State 3 to 5 and from State 4 to 6. The effect of dephosphorylation-induced uncoupling on P_o and ATP sensitivity may be only apparent in the

absence of PIP₂-dependent regulation (transitions from State 2 to 4 to 6) and have minor effects when PIP₂ regulates channel activity (transitions from State 1 to 3 to 5). Dephosphorylation may also have a long-term effect and cause deactivation of a PIP₂ regulatory site, facilitating the component of slow rundown related to loss of PIP₂ (pathway C). Lastly, not shown in this scheme, further rundown of States 5 and 6 leads to complete and irreversible channel closure, possibly after extensive Kir6.2 dephosphorylation. (B) In addition to decreasing the sensitivity to ATP by increasing P_o , PIP₂ may affect directly the binding of ATP to Kir6.2. This dramatic effect (sensitivity to ATP as low as 5 mM) occurs after prolonged exposure to PIP₂ and is independent of the rundown state of the channel. Under physiological conditions, such a decrease in binding affinity may be restricted to States 2–5, so that K_{ATP} channels remain sensitive to adenine nucleotides.

ysis (pathway C), another may involve channel dephosphorylation leading to uncoupling of SUR1 from Kir6.2 (pathway B). In Fig. 11, we show pathway B to follow MgADP dissociation (pathway A). We have no experimental evidence in favor of this specific scheme and the two pathways are shown as sequential only for clarity. In fact, the observation that in two patches channel rundown occurred within 2–4 min after patch excision even in the presence of MgADP, and ADP was inhibitory afterward, suggests that process A (dissociation of MgADP) and process B (dephosphorylation-induced uncoupling) can occur simultaneously. In other words, MgADP would not protect from dephosphorylation-induced uncoupling. This hypothesis is supported by our observation that dephosphorylation, induced experimentally by protein kinase inhibition, causes pronounced channel inhibition in the presence of MgADP. This marked inhibition was presumably a result of SUR1 uncoupling from Kir6.2, which unmasked the inhibitory effect of ADP binding to the ATP inhibitory site on Kir6.2. Such an effect would be most prominent when intracellular PIP_2 is low and has little effect on channel activity (transition from State 2 to 4 to 6). In contrast, when PIP_2 controls channel opening (high P_o), uncoupling due to dephosphorylation may have little effect on channel activity and sensitivity to ATP (transition from State 1 to 3 to 5). This hypothesis is supported by our observation that protein kinase inhibitors had almost no effect on channel activity after PIP_2 treatment (data not shown). However, after inducing loss of MgADP-dependent reactivation, long exposures to the protein kinase inhibitors also caused deactivation of the PIP_2 regulatory site on Kir6.2. Thus, dephosphorylation-induced rundown may involve two irreversible processes, one relatively fast and mediated by uncoupling of SUR1 from Kir6.2, and a slower one, involving deactivation of the PIP_2 regulatory mechanism. The latter indicates that loss of PIP_2 (pathway C) may, therefore, occur as a result of hydrolysis as well as dephosphorylation of Kir6.2. Finally, complete dephosphorylation of Kir6.2 may lead from either State 5 or 6 to a final state characterized by complete and irreversible channel closure (not shown).

Our data also indicate that prolonged exposure to PIP_2 causes a dramatic decrease in ATP sensitivity, without noticeable change in channel activity. This decrease in sensitivity to ATP evoked by PIP_2 is observed before as well as after prolonged and irreversible rundown. Before irreversible channel rundown, the main decrease in sensitivity to ATP occurs long after channel stimulation has reached saturation (Shyng and Nichols, 1998). Due to the extreme nonlinearity observed between ATP sensitivity and P_o when P_o is near maximum (Trapp et al., 1998; Enkvetchakul et al., 2000), PIP_2 -induced decrease in ATP sensitivity may be explained, in

this case, by minimal increase in P_o . However, such a mechanism cannot account for the dramatic decrease in ATP sensitivity observed with prolonged exposure to PIP_2 after irreversible channel rundown, since P_o remains very low. To account for this effect, we propose, in accordance with Fan and Makielski (1999), that PIP_2 has also a direct inhibitory effect on ATP binding to Kir6.2. This effect due to prolonged exposure to PIP_2 would occur independently of the channel state (Fig. 11 B). After slow rundown, the decrease in ATP affinity would lead the channel into States 5' and 6'. A similar effect of PIP_2 may occur before slow rundown that would lead to States 3' and 4'. It remains to be determined whether this effect of PIP_2 on adenine nucleotide binding to K_{ATP} channels is physiological, since K_{ATP} channels must be closed by intracellular ATP. It may therefore be postulated that intracellular PIP_2 levels are too low to cause adenine nucleotide desensitization. Alternatively, interaction of SUR with Kir6.2 may prevent this effect of PIP_2 in native channels. For these reasons, process D is not shown to have any effect on States 1 and 2.

In summary, MgADP, PIP_2 , and subunit phosphorylation have complex regulatory effects on Kir6.2+SUR1 and native K_{ATP} channels. They all stimulate channel activity and decrease ATP sensitivity to physiological levels. However, MgADP appears to have short-term effects, while PIP_2 and subunit phosphorylation regulate channel activity over longer time courses. Thus, MgADP is more likely to regulate acute cell function such as glucose-induced β -cell membrane depolarization, which occurs on a time scale of minutes, whereas PIP_2 and phosphorylation may be more important in long-term regulation relevant to physiological adaptation and/or pathophysiological conditions. Finally, with physiological MgADP present, intracellular PIP_2 may set the channel sensitivity to ATP near 400 μ M when high and 150 μ M when low.

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